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Application of Ionic Liquids based Microwave-Assisted Extraction to Bioactive Compounds Comfrey *(Symphytum officinale L.)* Leaves

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A B S T R A C T

Extraction of bioactive compounds from comfrey leaves (Symphytum officinale L.) was carried out by comparing organic solvents and ionic liquids between conventional and microwave-assisted extraction (IL-MAE) methods. The comfrey leaf powder was extracted under various conditions. The types of organic solvents used are 65% ethanol and ionic liquids. The extraction methods used are reflux and microwave-assisted extraction. The ionic liquid was extracted by microwave-assisted extraction with the following conditions: ionic liquid concentration of 1 mol/L, the solid-liquid ratio of 1:20 (g/mL), extraction time of 10 minutes, and power of 30% (270 Watt). The total phenolic content of the extract was analyzed with a microplate reader. Allantoin and retrorsine N-oxide levels were analyzed by thin-layer chromatography – densitometry. MAE extraction in a solvent containing 65% ethanol resulted in the highest total phenolic content of 1.038±0.012. The highest levels of allantoin were also obtained from MAE extraction with 65% ethanol solvent at 2.922 ± 0.123. The highest levels of retrorsine N-oxide were obtained by extraction of MAE with [BMIM]Br with a concentration of 0.049 ± 0.007. Extraction of comfrey leaves using the MAE method of 65% ethanol produced the highest total phenolic and allantoin content compared to other methods. [BMIM] Br extraction with MAE can attract the highest retrorsine N-oxide in comfrey leaves.

Kata kunci:

Symphytum officinale L.
Cairan ionic
Microwave assisted extraction
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A B S T R A K

Ekstraksi senyawa bioaktif daun komfrey (*Symphytum officinale* L.) dilakukan dengan membandingkan pelarut organik dan cairan ionik, antara metode konvensional dengan metode *microwave assisted extraction.* Serbuk daun komfrey diekstraksi dengan berbagai kondisi. Jenis pelarut organik yang digunakan yaitu etanol 65% dan cairan ionik. Metode ekstraksi konvensional yang dilakukan, yaitu refluks dan *microwave-assisted extraction***.** Cairan ionik diekstraksi dengan*microwave-assistedextraction* dengan kondisi: konsentrasi Cairan ionik 1 mol/L, Rasio padatcair 1:20 (g/mL), waktu ekstraksi 10 menit dan power 30% (270 Watt). Kadar fenol total dalam ekstrak dianalisis dengan *microplate reader*. Sedangkan kadar allantoin dan retrorsine *N-oxide* dianalis dengan kromatografi lapis tipis – densitometri. Ekstraksi MAE dengan pelarut etanol 65% menghasilkan kadar fenol total tertinggi, yaitu 1,038 ± 0,012. Kadar Allantoin tertinggi didapatkan juga dari ekstraksi MAE dengan pelarut etanol 65% sebanyak 2,922 ± 0,123. Kadar retrorsin *N*-oksida tertinggi didapatkan dengan ekstraksi MAE dengan [BMIM]Br dengan konsentrasi 0,049 ± 0,007. Ekstraksi daun komfrey dengan metode MAE etanol 65% menghasilkan kadar fenol total dan allantoin paling tinggi dibandingkan dengan metode lain. Ekstraksi [BMIM] Br dengan MAE dapat menarik kadar retrorsin *N*-oksida paling tinggi dalam daun komfrey.

INTRODUCTION

Comfrey (*Symphytum officinale* L.) is a plant from the Boraginaceae family used as herbal medicine for more than 2.000 years. Comfrey has been traditionally used to treat muscle pain due to inflammation and joint problems, respiratory problems, gastro intestinal diseases, metrorrhagia, phlebitis, and tonsillitis(Kruse et al., 2019; Rode, 2002; Staiger, 2012; Trifan et al., 2018).Comfrey is used topically, mainly for treating wounds, joint disorders, and musculoskeletal injuries of all kinds (Nastić et al., 2020).Comfrey leaves, herbs, and root extract have long been used to treat musculoskeletal disorders, wounds, gout, hematomas, and thrombophlebitis(Salehi et al., 2019).

Comfrey contains various chemical constituents, including allantoin, rosmarinic acid, mucilage, and pyrrolizidine alkaloids. The pyrrolizidine alkaloids such as retrorsine *N-oxide*, symphytine, lycopsamine, intermedine, acetyllintermedine, and acetyllycopsamine have been reported in comfrey(Trifan et al., 2018; Wynn & Fougère, 2007). In the other hand, pyrrolizidine alkaloids are metabolized in humans to hepatotoxic and pulmotoxic pyrrole derivatives in reactions catalyzed by CYP3A4(Janeš & Kreft, 2014). Comfrey's total pyrrolizidine alkaloid concentration was determined to range 1.380-8.320 µg/g root and 15–55 µg/g leaf(Salehi et al., 2019).In another study, the symphytine and echimidine content in comfrey leaves varied considerably between suppliers (Oberlies et al., 2004). Comfrey's anti-inflammatory and analgesic therapeutic properties are related to the compounds allantoin and rosmarinic acid, the molecular mechanism of action of these compounds has not been fully understood. Several cellular mechanisms, including targeting several intracellular signalling pathways triggered by inhibiting NF-κB signaling at two stages, have been presented to explain their mode of action (Seigner et al., 2019).

Rosmarinic acid is present in the family Boraginaceae. Rosmarinic acid has antioxidant, antiviral, and anti-inflammatory effects and shows a shallow potential for toxicity and antitumor effects(Savić et al., 2015). Allantoin is a heterocyclic purine derivative widely used, mainly because of its tissue repair properties, it is safe and non-toxic(Becker et al., 2010). Allantoin also has antioxidant, anti-inflammatory, pain-relieving, and gastroprotective effects. In addition, allantoin's ability to reduce the inflammatory process is associated with restoring enzymatic and nonenzymatic antioxidant pathways(Aman et al., 2021; Becker et al., 2010; da Silva et al., 2018).

Modern extraction techniques using green solvents have been developed to effectively and efficiently extract bioactive substances. Various procedures include the use of green solvents such as ionic liquids. Ionic liquids are composed of relatively large organic cations and either single or many anions. Ionic liquids are gaining significant interest due to their unique features, which make them appropriate for a variety of applications, including the extraction of bioactive chemicals from plants. The structure of an ionic liquid has a substantial effect on its physicochemical characteristics and is therefore predicted to have an effect on the liquid's extraction effectiveness for the target analyte. (Yang et al., 2011).Ionic Liquids (IL) based Microwave-assisted Extraction (MAE) is a non-conventional extraction method, that has been developed where the principle of IL-MAE is identical to that of the MAE technique, except that the focus and kind of solvent utilized are different (Ahmad, 2018).

Various analytical methods are used to discover bioactive constituents in medicinal plant materials(Shanaida et al., 2020).TLC detection of secondary metabolites from plant extracts is a widely used technique. For example, TLC densitometric techniques were employed to quantify allantoin and retrorsine *N-oxide* in a comfrey extract(Janeš & Kreft, 2014; Kimel et al., 2019; Mroczek et al., 2006; Smyrska-Wieleba et al., 2017). Additionally, the 96-well microplate reader technique has been widely applied to determine the total phenolic content of medicinal plants (Islamudin Ahmad et al., 2017).

The purpose of this research was to examine the effectiveness of conventional and ionic liquid (IL) extractions of chemicals from comfrey leaves. Allantoin and retrorsine N-*oxide* contents in comfrey leaf extract were quantified using thin layer chromatography-densitometry. Additionally, we determined the total phenolic content using a 96-well microplate reader method. According to the study, phytochemicals from comfrey leaves may be extracted using an extraction process based on the IL-MAE method.

METHOD

Chemicals and Materials

The sample used is leaf of comfrey plant. Samples were obtained from farmers in Karanganyar, Surakarta, Central Java. Comfrey was determined macroscopically at the Center for Biological Research, Gadjah Mada University. Standard retrorsine*N-oxide*, allantoin (Phytolab, Germany) and gallic acid (Merck, Germany). As shown in Table 1, this study used a total of eight distinct kinds of ionic liquids. (Shanghai Cheng Jie Chemical, China), Methanol pro analysis, Dichloromethane pro analysis, Ethanol pro analysis, Follin-Ciocalteu, Potassium dihydrogen phosphate (KH_2PO_4) pro analysis, sodium carbonate (Na_2CO_3) pro analysis, Acetonitrile pro analysis, 25% ammonia solution (Merck, Germany), Aquadest, Ethanol, Dichloromethane (Chemstation Asia) and TLCsilica gel60 F_{254} (Merck, Germany), Aquabidestilata and deionized water were obtained from the local supplier.

Instrumentation

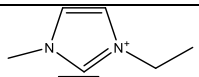
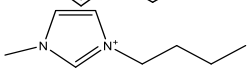
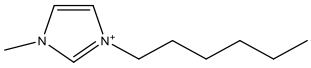
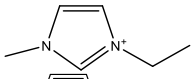
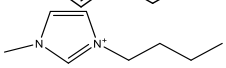
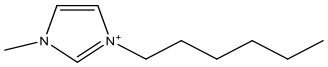
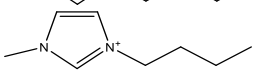
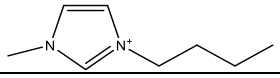
The equipment used in this study were analyte scales, reflux, hotplate, microwavewith modification (Modena MV 3002 900-Watt), Vacuum rotary evaporator (Buchi), a microfilter (Whatman 0,45 µm), Centrifuge EBA 200, Vortex WiseMix VM-10 (Daihan Scientific, Korea), 20-200 µl micropipette, 100 – 1000 µl and 0,5 – 5ml macropipette (Socorex), Twin Chamber Camag, UV lamp Camag, TLC Scanner IIIWinCATs system (Muttenz, Switzerland), refrigerator, vacuum drying oven, 96-wellplate (Iwaki), microplate reader (Versamax Microplate Reader, USA).

Conventional Reflux method

The reflux extraction method of comfrey leaf powder was conducted, according to Trifan et al., 2018 with slight modification. Five grams of comfrey leaf powder were extracted with 500 ml of 65% ethanol for 2 hours under reflux. The liquid extract then filtered using Whatman paper no. 1 (0.45 µm) and evaporated in a vacuum rotary evaporator at 40-45 ºC and concentrated in a water bath at

45 ºC. The extract was stored at -20 ºC until analysis was carried out.

Table 1.
Chemical structure of Ionic Liquids in this study

ILs	Cation	Anion
[EMIM]Br		Br^-
[BMIM]Br		Br^-
[HMIM]Br		Br^-
[EMIM]BF_4		BF_4^-
[BMIM]BF_4		BF_4^-
[HMIM]BF_4		BF_4^-
[BMIM]Tos		Tos^-
[BMIM]PF_6		PF_6^-

MAE method

Two grams of dried powder was placedinto a round bottom flask then added 40 ml of 65% ethanol. The flask was placed in the microwave at 30% power (270 Watt) for 10 minutes. The liquid extract obtained was filtered with Whatman paper (0.45 µm) and evaporated in a water bath at 45 ºC. The extract was stored at -20 ºC for further analysis.

IL-based MAE Method

The IL-MAE method was conducted based on a previous study with slight modification (Ma et al., 2010). Two grams of comfrey leaf powder was placed into a round bottom flask, then added 40 ml of ionic liquid. The flask was placed in the microwave at 30% power (270 Watt) for 10 minutes. The liquid extract obtained was filtered with Whatman paper (0.45 m filter). A total of 3 ml of the ionic liquid extract and 3 ml of KH_2PO_4 salt were put into a centrifuge tube, then vortexed for 10 seconds and centrifuged for 10 minutes at a speed of 3.000 rpm. A total of 3 ml of dichloromethane was added to the solution mixture, then vortexed for 10 seconds and centrifuged for 10 minutes at a speed of 3.000 rpm. The tube's fraction of dichloromethane in the lower phase was accommodated in an empty vial and evaporated in a water bath at 40ºC. The extract was stored at -20ºC for further analysis.

Experimental Procedures

Determination of Total Phenolic Content

The microplate total phenolic content Folin-Ciocalteau method was based on slight modifications from Ahmad et al. 2017 and Sembiring et al. 2017. Gallic acid standards were prepared in dilution concentrations of 6.25 - 200 µg/ml. A total of 25 µl of standard or sample was pipetted into a 96-wellplate, then 100 µl of 25% Folin Cociealteu was added and homogenized for 60 seconds. The mixture was incubated for 4 minutes at room temperature in the dark. A total of 75 µl of 10% Na_2CO_3 was added to the solution, shaken again for 60 seconds, and incubated for 2 hours at room temperature in the dark. The absorbance was measured at a wavelength of 765 nm using a microplate reader.Total phenol content was expressed as mg of Gallic Acid Equivalence (GAE) per gram of comfrey leaf powder(Ahmad et al., 2017; Sembiring et al., 2017).

Determination of Retrorsine N-Oxide Content

The determination of retrorsine N-oxidecontent was carried out by thin layer chromatography (TLC) method from Mroczek et al., 2006 with a slight modification. A standard solution of retrorsine *N-oxide* containing up to 2 mg was dissolved in 10 ml of methanol to achieve a concentration of 200 µg/ml. Retrorsine *N-oxide* standard is prepared in dilution concentrations of 25 - 200 µg/ml.

The sample or standard was spotted 10 µl on a TLC plate with the silica plate 1 cm from the bottom of the plate and 0.5 cm apart from each *sample*. The TLC plate was developed with dichloromethane, methanol, and 25% ammonia (100:20:4) v/v/v as mobile phases as far as 8.5 cm in a saturated chamber. The TLC plate was evaluated using a TLC scanner equipped with a 220 nm wavelength to determine the area under the curve (AUC).Data was processed with the Camag WinCats 1.4.8 program. Each plate was measured three times.

$$\text{CRetrorsine } \textit{N-oxide} = \frac{\text{sample area}}{\text{standard area}} \text{ x } \frac{\text{standard volume spotted}}{\text{sample volume spotted}} \text{x C Standard}$$

Determination of Allantoin Content

Determination of Allantoin content was carried out by the thin layer chromatography (TLC) method from(Kimel et al., 2019), with slight modifications. First, 5 mg of allantoin standard was dissolved in 5 ml of aquabidestilata to produce a standard solution of 1000 µg/ml. Allantoin standards are prepared in dilution concentrations of 50 - 1000 µg/ml. Standards or samples were spotted at 10 µl on a TLC plate and developed in the mobile phase of butanol: methanol

50%: formic acid (66,5:33,2:0,3) v/v/v for 8.5 cm in a saturated chamber. The TLC plate was dried in the oven at 120 ºC for 20 minutes. The TLC plate was evaluated using a TLC scanner equipped with a 200 nm wavelength to determine the area under the curve (AUC). Data was processed with the Camag WinCats 1.4.8 program. Each plate was measured three times.

$$\text{C Allantoin} = \frac{\text{sample area}}{\text{standard area}} \times \frac{\text{standard volume spotted}}{\text{sample volume spotted}} \times \text{C Standard}$$

Data analysis

The **extract's % yield was calculated using the formula:**

$$\text{Percentage yield (\%)} = \frac{\text{dry extract of comfrey}}{\text{dry powder of comfrey}} \times 100$$

All observations were made in triplicate for this study, and results were presented as Mean ± Standard Error of Measurement. The data were analyzed using the Analysis of Variance (ANOVA) method ($p < 0.05$), and the means were separated using Bonferroni's multiple comparison tests, both of which were performed using the program GraphPad Prism 8.

RESULTS AND DISCUSSION

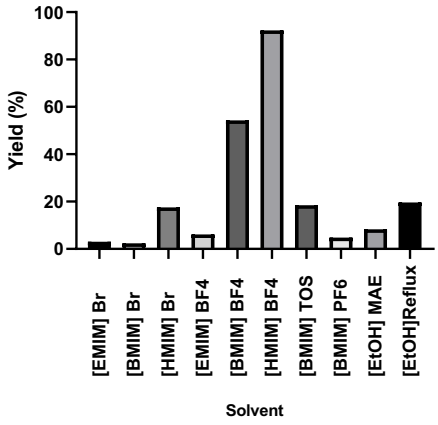


Figure 1: Yieldsof extract from various extraction

The extraction method was carried out by conventional methods with reflux and non-conventional by MAE, and the yield of all extracts isshown in Figure 1. When compared to other methods, the microwave-assisted extraction (MAE) method is the most acceptable for using an ionic liquid solvent. Non-conventional approaches, on the other hand, have been shown to be more successful and efficient in extracting the active constituents of plants(Ahmad et al., 2017). The different extraction methods aim to obtain an optimal extraction method with secondary metabolite targets. The extraction results obtained differences in theyields of extract. The highest yield was obtained from the [HMIM]BF_4at 92.28% and the lowest IL from [BMIM]Br at 2.38%. On the other hand, the 65% ethanol conventional solvent yield using the reflux method was 19.63% and with MAE, 8.28%.Due to the molecular structure of its anion and cation, ionic liquid is an enhanced extraction solvent. The length of the alkyl chain of the ionic liquid cation has a beneficial effect on the extraction yield but increases the hydrophobicity of the ionic liquid as the alkyl chain length increases. As a consequence of the correct hydrogen bonding and hydrophobic interactions, the solvation interactions with bioactive chemicals were improved (Xu et al., 2012).

Total Phenolic Content

A 96-well microplate reader was used to produce standard calibration curves. Based on linear regression analysis, Y = 0.0086x + 0.0418 and a correlation coefficient (R^2) of 0.9995 (Figure 2). Y is absorbance of gallic acid and, X is the standard concentration or sample. Equations are used to calculate the total phenolic content of comfrey leaf extracted using a variety of solvents and techniques.

The yields of total phenolic content varied according to the absorbance measured on the sample (shown in Table 2). The highest total phenolic content yieldswere produced using the MAE extraction method with 65% ethanol (1.038 ± 0.012 mgGAE/gdried comfrey). Extraction using MAE with 65% ethanol to the total phenolic content obtained was higher than the reflux solvent of 65% ethanol. This result was possible due to the suitable ratio of solvent and powder during microwave extraction, which resulted in inhomogeneous and effective heating(Chan et al., 2011). The MAE extract produced with all IL had much fewer or no phenolic compounds than ethanol.In this study, the separation of ionic liquids was carried out with dichloromethane as a solvent, which is less polar than ethanol.This result also confirmed that phenolic contents are more attracted to ethanol solvents than less polar solvents such as dichloromethane(Nastić et al., 2020). When selectivity and solubility are highly composition-dependent, these characteristics are also affected by the extraction temperature and microwave irradiation(Canales & Brennecke, 2016).

Table 2.
A summary of the extraction processes and a comparison of the levels

Solvent	Extraction Method	Total Phenolic Content (Mg GAE/ g dried comfrey)	Allantoin Content (mg/g dried comfrey)	Retrorsine *N-oxide* Content (mg/g dried comfrey)
[EMIM]Br	MAE	0.00664± 0.001	0.869 ± 0.113	0.018± 0.001
[BMIM]Br	MAE	0.01672 ± 0.001	1.335 ± 0.243	0.049± 0.007
[HMIM]Br	MAE	0.0003 ± 0.001	1.070 ± 0.240	-
[EMIM]BF_4	MAE	0.0094 ± 0.001	-	-
[BMIM]BF_4	MAE	-	-	-
[HMIM]BF_4	MAE	-	-	-
[BMIM]Tos	MAE	0.01092± 0.001	-	-
[BMIM]PF_6	MAE	-	-	-
65%Etanol	Reflux	0.891 ± 0,008	1.989 ± 0,574	-
65 %Etanol	MAE	1.038 ± 0.012	2.922 ± 0.123	-

Furthermore, the results obtained using the MAE technique with eight different types of IL showed that [BMIM] Br is capable of extracting much greater levels of phenolic content (0.01672 ± 0.001 mg/g dried comfrey) than other types of ionic liquids.According to the extraction yields with the [BMIM] cation, the anions Br^- and Tos^- were more efficient than other anions in the MAE of phenolic contents from comfrey leaves.The studies suggested that increasing the length of the alkyl chain has an effect on the extractionof the same Br^-. While the hydrogen bond acidity of the three cations increased proportionally as the alkyl chain length went from ethyl to hexyl at the site of the 1-alkyl-3-methylimidalizium ring, the hydrophobicity did not. [BMIM]Br's hydrogen bonding and hydrophobic contacts resulted in enhanced solvation interactions with phenolic compounds, which resulted in better extraction yields when compared to [EMIM]Br and [HMIM]Br(Du et al., 2009).

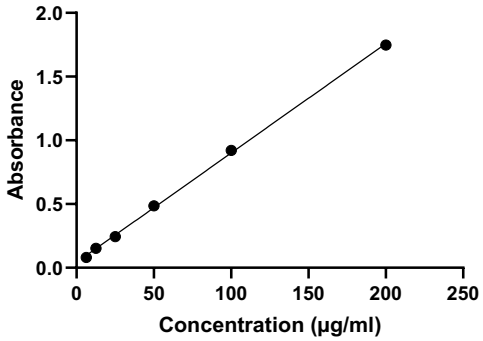


Figure 2: Curve of linear regression analysis of gallic acid

Allantoin Content

Standard calibration curves were performed using TLC-densitometry. The curve of the linear and chromatogram of allantoin is shown in Figure 3. Based on the analysis, the allantoin Rf value is 0.56 with linear regression obtained with the equation Y = 12.139x + 693.97. The correlation coefficient (R^2) is 0.9848 (Figure 3a). Y is the area of allantoin and X is the standard or sample concentration. Equations were applied to determine the allantoin content of comfrey leaf extract using various solvents and extraction methods.

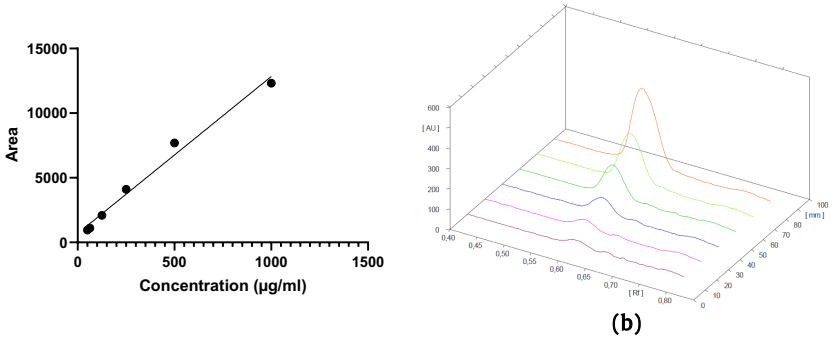


Figure 3: (a) Curve of linear regression analysis of Allantoin (b) Chromatogram of standar Allantoin

Allantoin contentwas determined by TLC and analyzed by a TLC scanner. The allantoin yields of each extract are presented in Table 2. The highest allantoin yields were produced using the MAE extraction method with 65% ethanol (2.922 ± 0.123mg/g dried comfrey).This result confirmed that allantoin content with ethanol is effective for extracting allantoin from comfrey (Kimel et al., 2019). Furthermore, the results obtained using the MAE technique with eight different ILs showed that Br^- anions with different cations from [EMIM] to [HMIM] were able to extract allantoin. [BMIM]Br was more efficientat extracting allantoin content (1.335 ± 0.243 mg/g dried comfrey) than other types

of ionic liquids.The cations, particularly the anions, of IL had an effect on the extraction, and ILs electron-rich aromatic π-system facilitated in extraction (Du et al., 2009).

RetrorsineN-oxide Content

Retrorsine N-oxide standard calibration curve was performed using TLC-densitometry. The curve of the linear and chromatogram of retrorsine *N-oxide* show in Figure 4. Based on the analysis, the retrorsineN-oxideRf value is 0,4 with linear regression obtained with the equation Y = 33.215X– 738.66 and the correlation coefficient (R^2) is 0.9886. Y is the area of Retrorsine N-oxide and X is the standard or sample concentration. Equations were applied to determine the Retrorsine N-oxidecontent of comfrey leaf extract using various solvents and extraction methods.

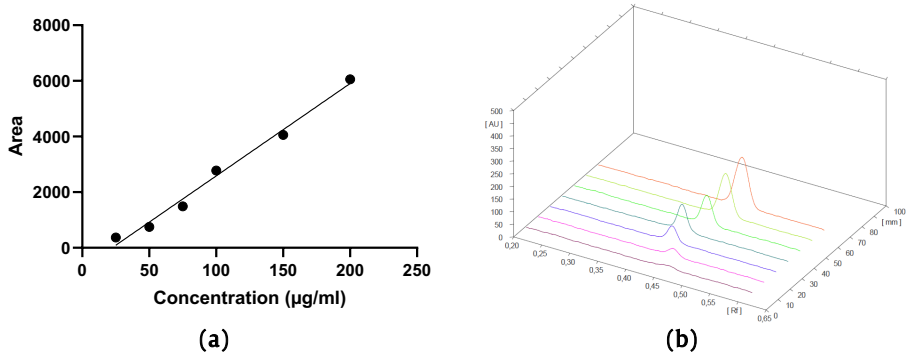


Figure 4: (a) Curve of linear regression analysis of retrorsine *N-oxide* (b) Chromatogram of standar retrorsine *N-oxide*

Determination of retrorsine *N-oxide* content was carried out by TLC and analyzed by TLC scanner. Retrorsine N-oxideyields of each extract are presented in Table 2. Retrorsine *N-oxide* yields were only obtained from extracts of [BMIM]Br (0.049 ± 0.001 mg/g sample) and [EMIM]Br (0.018 ± 0.001 mg/g sample).The extraction efficiency of retrorsine N-oxide obtained with [BMIM]Br aqueous solution was significantly greater than the other IL, indicating that [BMIM]Br was more effective than the other IL-MAE of retrorsine *N-oxide*. This is probable because [BMIM]Br exhibited more multi-interactions with alkaloids, including π–π, ionic/charge–charge, and hydrogen bonding. Additionally, [BMIM]Br exhibited a little greater acidity, which may aid in the extraction of the target pyrrolizidine alkaloid from comfrey leaves(Ma et al., 2010).The separation of the ionic liquid is carried out with dichloromethane as a solvent, which has fewer polar properties than ethanol. Pyrrolizidine alkaloid compounds, one of which is retrorsine N-oxide, can be extracted using chloroform or dichloromethane as a solvent. This is confirmed in previous studies, the use of dichloromethane resulted in higher extraction of less polar or non-polar compounds than ethanol solvents(Kopp et al., 2020; Nastić et al., 2020).The ethanol extract using MAE and the reflux method did not show any retrorsine N-oxide content. This is possible because the retrorsine content of the ethanol extract is very small, so it cannot be detected using the tlc-densitometry method. The pyrrolizidine alkaloids in the previous study were extracted with methanol/ethanol as a solvent, which was added with an acid such as tartaric acid, ascorbic acid, and hydrochloric acid, or by the addition of a base such as ammonia(Kopp et al., 2020).

CONCLUSION AND SUGGESTION

The results obtained concluded that the extraction of comfrey leaves with 65% ethanol solvent MAE method produced the highest total phenolic and allantoin content compared to other methods. In addition, extraction of ionic liquid [BMIM]Br with MAE resulted in the highest content of retrorsine N-oxide compared to other methods.

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Conflict of interest

This research does not include any conflict of interest

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